Differential chromatin structure within a tandem array 100 kb upstream of the maize *b1* locus is associated with paramutation

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Recombination mapping defined a 6-kb region, 100 kb upstream of the transcription start site, that is required for *B-I* enhancer activity and paramutation—a stable, heritable change in transcription caused by allele interactions in maize (*Zea mays*). In this region, *B-I* and *B′* (the only *b1* alleles that participate in paramutation) have seven tandem repeats of an 853-bp sequence otherwise unique in the genome; other alleles have one. Examination of recombinant alleles with different numbers of tandem repeats indicates that the repeats are required for both paramutation and enhancer function. The 6-kb region is identical in *B-I* and *B′*, showing that epigenetic mechanisms mediate the stable silencing associated with paramutation. This is the first endogenous gene for which sequences required for paramutation have been defined and examined for methylation and chromatin structure. The tandem repeat sequences are more methylated in *B-I* (high expressing) relative to *B′* (low expressing), opposite of the typical correlation. Furthermore, the change in repeat methylation follows establishment of the *B′* epigenetic state. *B-I* has a more open chromatin structure in the repeats relative to *B′*. The nuclease hypersensitivity differences developmentally precede transcription, suggesting that the repeat chromatin structure could be the heritable imprint distinguishing the two transcription states.

[*Key Words*: Epigenetics; chromatin; DNA methylation; transcriptional enhancer]

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Paramutation is an interaction between specific alleles that causes a meiotically heritable change in one allele's expression. Paramutation was originally defined as an interaction between two alleles (Brink 1956), but paramutation has also been shown between a transgene and a homologous endogenous gene in nonallelic positions (Sidorenko and Peterson 2001). Features are shared with *trans*-silencing phenomena in *Drosophila* (Henikoff and Comai 1998) and fungi (Colot et al. 1996; van West et al. 1999) and with transcriptional gene silencing in plants (Chandler et al. 2000; Vaucheret and Fagard 2001) and animals (Garrick et al. 1998; Henikoff 1998). However, with few exceptions (Grewal and Klar 1996; Cavalli and Paro 1998), silencing is not meiotically heritable in fungi and animals. Expression of transposable elements, or genes with transposable elements, can be heritably changed by interactions with other elements (McClintock 1965; Krebbers et al. 1987; Martienssen 1996). Also, allele interactions between transposons alter the excision-repair mechanism (van Houwelingen et al. 1999). An eventual understanding of mechanisms underlying paramutation should reveal how alleles and other homologous sequences interact in the nucleus to influence the regulation of each other, and how heritable expression states are established, maintained through numerous cell divisions, and transmitted to the next generation.

Paramutation has been described for four maize (*Zea mays*) genes (*b1*, *r1*, *pl1*, and *p1*), all of which encode transcription factors that activate the biosynthesis of flavonoid pigments (Brink 1956; Coe 1959; Hollick et al. 1995; Sidorenko and Peterson 2001), and for genes in other plant species (for review, see Brink 1973). Paramutation at *b1* is extremely stable and the most penetrant of all systems described to date. The paramutagenic *B′* allele, which causes a lightly pigmented phenotype, arises spontaneously from the paramutable *B-I* allele, which confers a darkly pigmented phenotype, at a frequency of ~1%–10% (Coe 1966). Once formed, *B′* is extremely stable. In *B-I*/*B′* plants, *B-I* is always changed to *B′* (Coe 1966; Patterson et al. 1995). The new *B′* allele (which had been *B-I* in the previous generation) is indistinguishable from the parental *B′* allele in its ability to paramutate *B-I* in subsequent generations.

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Paramutation is associated with a 10- to 20-fold reduction in transcription of *B′* relative to *B-I* (Patterson et al. 1993). No differences in DNA sequences, rearrangements, or methylation patterns have been detected within 10 kb spanning the transcribed region of *B-I* and *B′* (Patterson et al. 1993, 1995). However, a quantitatively distinct DNase I hypersensitive site was observed in *B-I* and *B′* chromatin near the transcription start site (Chandler et al. 2000). Recombination experiments showed that sequences required for paramutation are tightly linked to *B′* (≤0.1 cM) and map upstream of the transcribed region (Patterson et al. 1995). Recent fine-structure recombination experiments mapped the *B′* sequences required for paramutation to a 13-kb region located ~100 kb upstream of the transcription start site (Stam et al. 2002). Herein we describe fine-structure recombination experiments that map *B-I* enhancer and paramutation activities to a 6-kb region within the 13-kb *B′* region. We compare this region in *B′* and *B-I* with two alleles that do not participate in paramutation and discuss models consistent with our findings.

Results

Isolation of recombinant B-I *alleles delimiting the sequences required for high expression*

Previously, the sequences required for *B-I* transcription were mapped upstream of the transcribed region (Patterson et al. 1995). To precisely map these sequences and determine their location relative to sequences required for paramutation, recombination experiments were performed using *B-I* and *B-Peru*. For simplicity, throughout the manuscript we refer to the region required for high expression as the enhancer. *B-I* plants are dark purple, whereas *B-Peru* plants are essentially green (Radicella et al. 1992). *B-I* participates in paramutation (it changes to *B′* when heterozygous with *B′*), whereas *B-Peru* does not (it is unchanged when heterozygous with *B′*; i.e., it is neutral). The two alleles are also distinguished by differences in seed expression: *B-Peru* produces purple seeds, whereas *B-I* produces colorless seeds. A 2.5-kb sequence in the promoter-proximal region of *B-Peru* confers seed expression (Radicella et al. 1992; Selinger et al. 1998). The seed pigment and unique 2.5 kb in *B-Peru* provide phenotypic and molecular markers for the promoter-proximal regions of the two alleles.

We hypothesized that a regulatory region containing a transcriptional enhancer, symbolized by E in Figure 1, functions in *B-I* and is either not present or not functional in *B-Peru*. Our strategy to map E is diagramed in Figure 1. Recombination events between E and the *B-I* transcribed region (Interval I) would transfer E to *B-Peru*, leading to an allele with high plant expression and the loss of E from *B-I*. The Interval I breakpoint occurring farthest upstream would define a 3′ boundary for E. Recombinants in Interval II should produce the same pigment phenotypes as the parental alleles, darkly pigmented *B-I* plants and nearly green *B-Peru* plants. The Interval II breakpoint located farthest downstream would define the 5′ boundary for E.

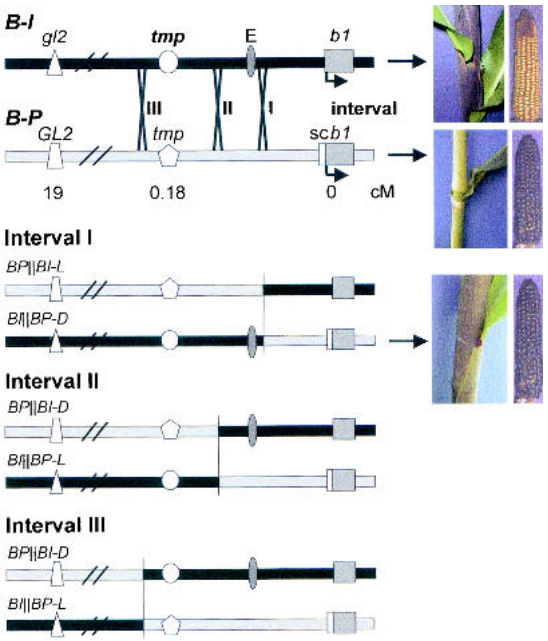

Figure 1. Recombination experiments with *B-I* and *B-Peru* (*B-P*) to map enhancer sequences. The gray oval (E) contains the hypothetical enhancer sequences. The phenotypic marker *glossy2* (*gl2*) and the molecular marker *tmp* were used to identify recombinants; polymorphisms are indicated with different symbols. Three recombination intervals between *gl2* and the *b1* coding region are indicated. The *B-Peru* promoter-proximal region contains sequences directing purple seed color (sc). *B-I* lacks these sequences. Vegetative and seed pigment phenotypes of *B-I*, *B-Peru*, and *BI‖BP-D1* (*D*, dark pigment; *L*, light pigment; ‖, recombinant allele with the parental allele contributing sequences upstream of the recombination breakpoint indicated on the left of the vertical bars and the parental allele contributing the coding and promoter proximal region indicated on the right) are shown.

To identify recombinants in Intervals I and II, we used the phenotypic *glossy2* (*gl2*) and molecular *tmp* markers. Plants containing *gl2 B-I* were crossed with *Gl2 B-Peru* plants, and the F_1 plants were crossed with *gl2 b-K55* plants, resulting in 50% colorless seeds (*B-I*/*b-K55*) and 50% purple seeds (*B-Peru*/*b-K55*). To first test if we could transfer the *B-I* enhancer to *B-Peru*, we planted 8910 purple seeds and scored the resulting plants for pigment levels. One plant with vegetative pigment much darker than *B-Peru* was identified (Fig. 1), confirmed to carry a recombinant allele using *tmp*, and designated *BI‖BP-D1* (D refers to dark). The ‖ symbol indicates a recombinant allele with the parental allele contributing sequences upstream of the breakpoint indicated left of the vertical bars and the parental allele contributing the promoter-proximal and coding regions indicated to the right of the vertical bars.

The isolation of *BI‖BP-D1* showed that recombination mapping could be used to localize sequences required for high expression in *B-I*. Subsequently, additional seeds were planted in the greenhouse, with 5677 colorless (*B-I* coding and promoter-proximal sequences) and 10,106

purple (*B-Peru* coding and promoter-proximal sequences) seeds germinating. Seedlings with a recombination event between *gl2* and *b1* (~20%) were transferred to the field, and leaf samples were screened via PCR for molecular markers (see Materials and Methods). Two Interval I recombinants were identified: the original allele isolated by screening for increased pigment levels (*BI‖BP-D1*) and a second allele (*BI‖BP-D2*). Both recombinants produced dark *B-Peru* plants, showing that enhancer sequences were transferred from the high-expressing *B-I* allele to the weakly expressing *B-Peru* allele. In all, 29 Interval II recombination events were identified: 23 *BP‖BI* recombinants were darkly pigmented (*BP‖BI-D*); 3 *BP‖BI* recombinants had a light, *B′*-like phenotype consistent with spontaneous paramutation of *B-I* to *B′*; and 3 *BI‖BP* recombinants had the *B-Peru* pigment phenotype, 2 of which set seed (*BI‖BP-L*; L for light). The Interval III recombinants identified (~4000) were not analyzed further.

Sequences required for high expression of B-I *are between 99 and 105 kb upstream of the transcription start site*

We used *B′* sequence information (Stam et al. 2002) to identify the most informative recombinants. Recombination breakpoints were mapped using DNA blot analyses with unique copy probes distributed throughout the 110-kb region upstream of the *B-I* transcription start site (Stam et al. 2002), and then designing primers to amplify DNA surrounding the breakpoints from the recombinant alleles. All fragments were cloned, sequenced, and compared with parental *B-I* and *B-Peru* sequences (Table 1).

The Interval I recombinant allele with a breakpoint at 99 kb (*BI‖BP-D1*) upstream of the *B-I* transcription start site defined the 3′ boundary. The darkly pigmented phenotype of this recombinant indicated the enhancer sequences had been transferred to *B-Peru* and were therefore mapped upstream of the breakpoint. The 5′ boundary was defined by the *BP‖BI-D1* and *BP‖BI-D2* alleles, which fully retained the enhancer function and have a recombination breakpoint 105 kb upstream of the *B-I* transcription start site.

Enhancer and paramutation sequences are contained in the same DNA region

Sequences required for paramutation in *B′* mapped between 93 kb and 106 kb upstream of the *B′* transcription start site (Stam et al. 2002), overlapping the region required for enhancer activity. We therefore asked which

Table 1. *Recombinants defining* B-I *sequences required for enhancer and paramutation activities*

				Paramutation phenotype[d]		
Allele	Interval[a]	Breakpoint[b] (kb)	Plant color[c]	Spontaneously paramutagenic	Expression suppressed by *B′*	Acquires paramutagenicity
BI‖BP-D1	I	99.3–99.4	Dark *B-Peru*	No (108)	Yes	Yes (114/118)[e]
BI‖BP-D2	I	97.7	Dark *B-Peru*	No (289)	Yes	Yes (589/589)
BI‖BP-L1	II	101.3–102.8	*B-Peru*	No (20)	N.A.	No (0/174)
BI‖BP-L2	II	105–105.2	*B-Peru*	No (37)	N.A.	No (0/66)
BP‖BI-D1	II	105	*B-I*	N.T.	Yes	Yes (38/38)
BP‖BI-D2	II	104.9–105	*B-I*	N.T.	Yes	Yes (71/71)
BP‖BI-D3	II	>108.5[f]	*B-I*	No (4/4)	Yes	N.T.
BP‖BI-D5	II	>108.5[f]	*B′*	Yes (54/54)	N.T.	N.A.
BP‖BI-D8	II	>108.5[f]	*B-I*	No (11/11)	Yes	Yes (42/42)
BP‖BI-D10	II	>108.5[f]	*B-I*	No (8/8)	Yes	Yes (18/18)
BP‖BI-D12	II	>108.5[f]	*B-I*	N.T.	Yes	N.T.
BP‖BI-D13	II	>108.5[f]	*B-I*	N.T.	Yes	Yes (35/35)
BP‖BI-D14	II	>108.5[f]	*B-I*	N.T.	Yes	N.T.
BP‖BI-D17	II	>108.5[f]	*B-I*	N.T.	Yes	N.T.
BP‖BI-D18	II	>108.5[f]	*B-I*	N.T.	Yes	Yes (30/30)

[a]Interval in which the recombination breakpoint mapped, based on molecular markers and pigment phenotypes.
[b]Recombination breakpoint defined by nucleotide polymorphisms between *B-I* and *B-Peru* (kilobases upstream of *B-I* transcription start site).
[c]Plant color produced by the homozygous recombinant allele.
[d]Ability of each allele to participate in paramutation. (Spontaneously paramutagenic) To determine if an allele was spontaneously paramutagenic it was crossed with *B-I* and progeny were scored for pigment phenotype. No, all progeny were *B-I;* Yes, all progeny were *B′*; N.T., not tested. The number of plants tested is in parentheses. (Expression suppressed by *B′*) To test if an allele was paramutable, it was crossed to *B′*. The phenotype of the F_1 was scored or the F_1 was self-pollinated and the pigment phenotype was scored in progeny homozygous for the recombinant allele; Yes, pigment levels were dramatically reduced; N.A. (not applicable), the allele produced no pigment. (Acquired paramutagenicity) To score if the allele became paramutagenic after being heterozygous with *B′*, the F_1 was crossed with *B-I* and progeny were scored for pigment. Yes, progeny had the *B′* phenotype; No, all progeny had the *B-I* phenotype; N.A., the allele was *B′*. The number of *B′* progeny/total tested is in parentheses.
[e]Four exceptions looked like *B-I* (see text for additional crosses).
[f]Recombinants with *B-I* polymorphisms up to at least 108.5 kb.

recombinant alleles retained *B-I* sequences required for paramutation (Table 1). The *BI‖BP-D1* and *BI‖BP-D2* alleles were not paramutagenic; they did not decrease *B-I* expression when heterozygous with *B-I*. To test if these alleles were paramutable (if their expression was reduced by *B′*), they were crossed to *B′*. To test if the alleles could become paramutagenic, the resulting F_1 plants were crossed to *B-I*. When heterozygous with *B′*, the *BI‖BP-D1* and *BI‖BP-D2* alleles became lightly pigmented and paramutagenic (Table 1). A similar result was obtained with the *BP‖BI-D* alleles. All of these alleles contained not only the *B-I* enhancer sequences, but also the sequences required for *B-I* to participate in paramutation. The two *BI‖BP-L* alleles that lacked the enhancer were not paramutagenic in crosses with *B-I* and did not become paramutagenic when crossed to *B′* and subsequently *B-I* (Table 1). Thus, the *B-I* enhancer and paramutation sequences map to the same 6-kb region, within the 13-kb region previously shown to be required for *B′* paramutagenicity (Stam et al. 2002).

B′ *and* B-I *are epialleles*

Comparison of the *B′* and *B-I* regions required for paramutation should show whether paramutation is associated with DNA sequence changes or not. We used information from *B′* (between 94 and 110 kb upstream of the transcription start site; Stam et al. 2002) to isolate the same region from *B-I* and two neutral alleles, *B-Peru* and *b-K55*. The resulting fragments were cloned and sequenced (see Materials and Methods). Extensive DNA blot analyses were done on leaf DNA from *B′*, *B-I*, *B-Peru*, and *b-K55* plants to verify the sequence contigs (data not shown).

B′ and *B-I* sequences between 94 and 110 kb upstream of the transcription start site are identical (accession nos. AY078063, AF475145, AF483657). DNA sequence comparisons of the promoter-proximal and coding regions of *B-I* and *B′* also revealed no differences between these two alleles (Patterson et al. 1993). Furthermore, extensive physical mapping using pulsed field gel electrophoresis (PFGE) and regular gel electrophoresis with DNA blot analyses revealed no differences between *B′* and *B-I* within a 220-kb region upstream of the transcribed region (Stam et al. 2002). These data indicate that *B-I* and *B′* are epialleles and paramutation is mediated by epigenetic mechanisms.

Sequences involved in paramutation contain tandem repeats

To investigate potential epigenetic mechanisms, we compared the 6-kb region of *B-I* and *B′* with two neutral alleles, *B-Peru* and *b-K55*. *B-I* and *B′* contain an 853-bp fragment that is repeated seven times in tandem, whereas both *B-Peru* and *b-K55* contain a single copy of this fragment (white and shaded arrows in Fig. 2A). The sequence is not repeated elsewhere in the genome. The 3.69-kb fragment in *B-Peru* that spans the single-copy 853-bp sequence is nearly identical to the same region in *B-I* and *B′* (Fig. 2A), suggesting that the repeated nature of this sequence in *B-I* and *B′*, rather than sequence polymorphisms, is mediating paramutation. We examined three other neutral alleles using DNA blots, and all three also had one copy of this region (data not shown).

The structure of three recombinant alleles provided a test of whether the repeats are involved in paramutation. *BI‖BP-L1*, *BI‖BP-D1*, and *BI‖BP-D2* contain 1, 3, and 5 repeats, respectively, whereas *B′* and *B-I* contain seven repeats (Fig. 2). A comparison of the paramutation strength of each of these alleles shows that the *BI‖BP-L1* allele with one repeat is neutral. In contrast, both alleles with multiple repeats, *BI‖BP-D1* and *BI‖BP-D2*, become paramutagenic when crossed to *B′*. The *BI‖BP-D1* allele with three repeats is less paramutagenic (4 exceptions in 118 plants; Table 1) than the *BI‖BP-D2* allele with five repeats (0 exceptions in 589 plants). When each of the four dark exceptions was crossed again to *B-I* and purple seeds (*BI‖BP-D1/B-I*) were planted, both *B′* and *B-I* plants were observed. The presence of *B′* plants indicated that the *BI‖BP-D1* allele retained paramutagenic activity. The

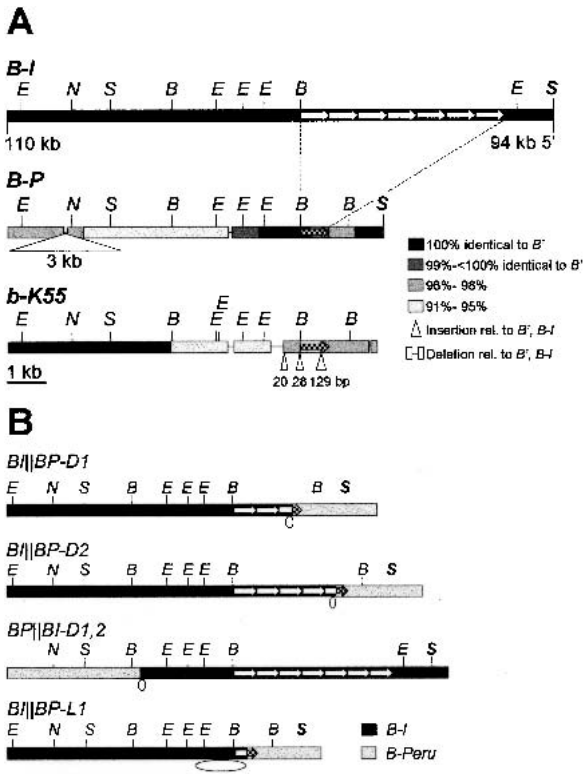

Figure 2. Sequence and structure of *B′*, *B-I*, *B-Peru*, *b-K55*, and recombinants. (*A*) Percentage identity between *B-I*, *B-Peru* (*B-P*), and *b-K55* as compared with *B′* is indicated by bar color. (*B*) Restriction maps of recombinant alleles defining 5′ and 3′ boundaries of *B-I* sequences required for enhancer and paramutation activities. The white ovals indicate the region in which recombination occurred. The arrows represent the tandem repeats (*B-I* is white, *B-Peru* and *b-K55* are shaded). Restriction sites are (B) *Bam*HI; (E) *Eco*RI; (N) *Nco*I; and (S) *Swa*I.

occurrence of even more *B-I* plants than what was observed in the first generation (2/18, 3/19, 12/104, 30/41) suggests that the paramutagenic state of *BI‖BP-D1* is unstable. The *BI‖BP-D1* allele was unstable even after multiple crosses to *B′*. When *BI‖BP-D1* was crossed to *B′* for two or three sequential generations and then crossed with *B-I*, 3/200 and 4/498 *B-I* plants were observed out of the total examined, respectively. In sharp contrast, no exceptions have ever been observed for the *B′* allele; when it is crossed to *B-I* only *B′* plants are observed with more than 100,000 plants tested in 50 years of study (Coe 1966; Patterson et al. 1993, 1995; E.H. Coe, Jr. and V.L. Chandler, unpubl.). The facts that the allele with one repeat is not paramutagenic and the allele with three repeats (two *B-I* and one hybrid repeat) has a reduced stability suggest that multiple repeats are required for paramutation.

Although all the repeats are nearly identical, the six sequence polymorphisms between *B-Peru* and the seven repeats in *B-I* and *B′* (Fig. 3) allowed us to compare where recombination occurred relative to these nucleotide polymorphisms in the alleles with differing numbers of repeats (Fig. 2B). In *BI‖BP-L1* the breakpoint is upstream of 220 bp (Fig. 3), resulting in retention of the six *B-Peru* polymorphisms. These same polymorphisms are retained in the hybrid fifth repeat in *BI‖BP-D2*, as its breakpoint was between 424 and 470 bp. The fact that *BI‖BP-D2* is fully paramutagenic supports the argument that the *B-Peru* repeat sequence polymorphisms alone are not what prevents paramutation activity.

Comparison of the structure of *BI‖BP-L1*, with no enhancer activity and containing one repeat and *B-I* sequences upstream (the *B-I* and *B-Peru* sequences in the first 220 bp of the first repeat and 1292 bp upstream are identical), with that of *BI‖BP-D1*, *BI‖BP-D2*, *BP‖BI-D1*, and *BP‖BI-D2*, which have multiple repeats and retain enhancer activity (Fig. 2B), indicates that multiple repeats are also needed for enhancer activity. Three repeats can mediate enhancer function, as there was no obvious pigment difference between *BI‖BP-D1* and *BI‖BP-D2* with three and five repeats, respectively. Both *BI‖BP-D1* and *BI‖BP-D2* have slightly less pigment than *B-I*, which could mean that seven repeats are needed for full enhancer activity. However, there could be additional sequences in *B-I* between the enhancer and promoter-proximal regions that contribute to the higher expression level; such a sequence was identified in *B′* (Stam et al. 2002). Also, the enhancer may not act as efficiently with the distinct promoter-proximal sequences in *B-Peru* relative to *B-I*.

Sequence characteristics of the tandem repeat in B-I *and* B′

The above studies show that multiple repeats are involved in paramutation and enhancer activity; therefore, we were interested in the tandem repeat's sequence properties. Sequencing identified a few sequence polymorphisms among the repeats in *B′*, but *B-I* had exactly the same polymorphisms. Figure 3 shows an alignment of the sequence found in *B-Peru* with a consensus of the seven repeats found in *B′* and *B-I*. The 60% AT-rich sequence with blocks of AT nucleotides interspersed with short blocks of high GC content appears to be noncoding and showed no significant identity with any known gene. Two sequences associated with epigenetic properties, yeast autonomously replicating sequences (ARS; Maundrell et al. 1988) and matrix attachment regions (MARs; Michalowski et al. 1999), are also AT-rich. We therefore examined the tandem repeat for the yeast ARS consensus and for sequence properties typical of MARs; numerous putative ARS and a putative MAR were identified (Fig. 3). Repeat finder programs identified short (~10–20 bp) inverted and direct repeats too numerous to display.

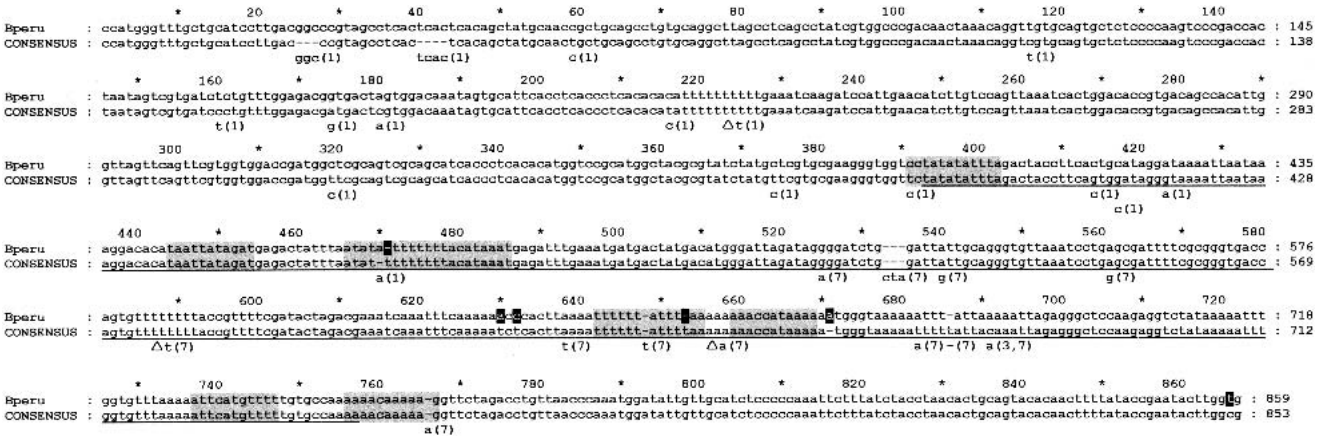

Figure 3. Sequence features of the tandem repeat in *B′* and *B-I*. Alignment of the *B-Peru* sequence with the consensus of the seven repeats in *B′* and *B-I*. Polymorphisms among the seven repeats are indicated below the consensus, the number in parentheses indicating in which repeat the polymorphism occurred (1 is most upstream, 7 is most downstream). Black highlights show positions of sequence divergence unique to the *B-Peru* repeat. Gray highlights show regions of similarity to yeast ARS, with one or two mismatches allowed from the 11-bp consensus, (A/T)TTTAT(A/G)TTT(A/T). The region of potential MAR similarity is underlined.

B-I *shows more DNA methylation within the repeated region than* B′

In higher plants and animals, differences in transcription states frequently correlate with cytosine methylation. Previous analyses did not detect differences in DNA methylation within *B′* and *B-I* in an ~10-kb region (the first 941 bp of the transcribed region plus 9 kb upstream; Patterson et al. 1993). To compare the methylation pattern in the sequences required for paramutation and enhancer activity in *B′* and *B-I*, genomic DNA was isolated from *B′*, *B-I*, and *B-Peru* leaves at three different developmental stages (two-week-old seedlings, just prior to and after tassel emergence) and from husk tissue on the first day that silks were visible. Each DNA sample was cut with *Eco*RI and *Sau*3AI (which does not cut its recognition site, GATC, when the C is methylated) or with *Eco*RI and *Mbo*I (a methylation-insensitive isoschizomer of *Sau*3AI). We examined 24 samples from 23 *B′* individuals, 30 samples from 26 *B-I* individuals, and 9 samples from 8 *B-Peru* individuals. No differences in methylation patterns were detected between the different developmental stages or tissues.

The *Sau*3AI sites flanking the fragments recognized by probe A served as a control for complete digestion as these sites were unmethylated in all genotypes (Fig. 4, probe A). There are three GATC sites within the 853-bp repeat (Fig. 4, labeled 1, 2 and 3), and when all sites are cut in *B′* and *B-I* DNA, fragments of 74, 296, and 483 bp are expected, but only the 296- and 483-bp fragments are seen along with the 189- and 397-bp fragments flanking the repeats (Fig. 4, *Mbo*I digests hybridized with the repeat probe C). When all sites are cut in *B-Peru* DNA, fragments of 397, 296, and 189 bp are observed (Fig. 4, probe C); eight of the nine *B-Peru* samples had unmethylated *Sau*3AI sites (Fig. 4, the *Sau*3AI and *Mbo*I patterns were identical). The one exception showed methylation at the third *Sau*3AI site (data not shown). Both *B′* and *B-I* samples have methylated *Sau*3AI sites in the tandem repeat region (Fig. 4, probe C). All *B′* and *B-I* samples showed methylation of the symmetric C in the third *Sau*3AI site (GATCTG), resulting in the 779-bp fragment. In 90% of the *B-I* samples (27/30) the asymmetric C in the second *Sau*3AI site (GATCCA) was methylated, generating the 376- and 853-bp fragments. The presence of the 296- and 779-bp fragments in *B-I* shows that some repeats are unmethylated at site 2. In contrast, none of the *B′* samples (0/24) showed detectable methylation of this site. The asymmetric site 1 (GATCCC) was generally cut in all repeats in both genotypes, but we occasionally saw a weak 557-bp band, representing methylation at this site.

To determine if the differential *B′* and *B-I* repeat methylation pattern extended to other regions, two other probes were used on the same DNA blots. All other sites showed equivalent methylation in *B-I* and *B′*. The *Sau*3AI sites monitored by probe B (Fig. 4, upstream of the repeats) were always unmethylated in *B-Peru* (9/9)

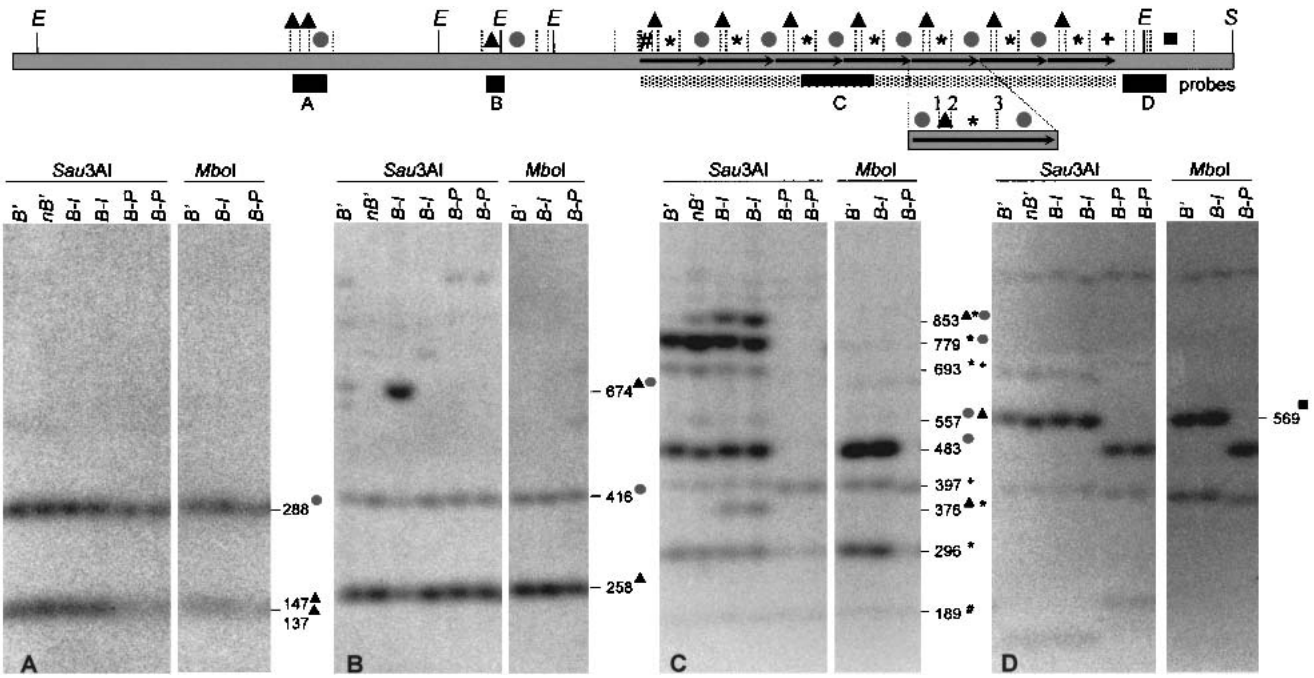

Figure 4. *B-I* DNA is more methylated within the tandem repeats than *B′* DNA. DNA blot analyses were done on *B′*, new *B′* (*nB′*), *B-I*, and *B-Peru* genomic DNA cut with *Eco*RI and *Sau*3AI (methylation-sensitive) or *Mbo*I (methylation-insensitive). Blots were hybridized with probes indicated on the *B-I*, *B′* map; the stippled bar indicates the region recognized by probe C. Arrows indicate tandem repeats. An enlargement of one repeat is shown. The restriction sites are (E) *Eco*RI; (S) *Swa*I. Vertical lines on the map indicate *Sau*3AI/*Mbo*I sites in regions recognized by the probes. Various symbols indicate fragments visible on blots; size is in base pairs. One blot hybridized with each indicated probe is shown.

and occasionally methylated in *B′* (6/24) and *B-I* (9/30) samples. A similar result was obtained with probe D located immediately downstream of the repeats. Most of the *B′* (22/24), *B-I* (25/30), and *B-Peru* (9/9) samples had a low level of methylation in this region; 2/24 *B′* and 5/30 *B-I* showed additional methylation. The only reproducible difference in methylation pattern between *B′* and *B-I* was at the second *Sau*3AI site in the repeats, suggesting it could be used as a molecular marker for the *B′* versus *B-I* epigenetic state.

The B′ *methylation pattern follows the establishment of the* B′ *epigenetic state*

To investigate the dynamics of the methylation pattern difference in the *B′* and *B-I* repeats, we next examined *B′* alleles that formed spontaneously. *B-I* spontaneously changes to *B′* at a relatively high frequency (Coe 1966; Patterson et al. 1995), and these events are seen as *B′* progeny arising from *B-I* parents. To determine whether a newly formed *B′* allele had the *B-I* (parental) or *B′* (present epigenetic state) methylation pattern, DNA was isolated from leaves (just before and after tassel emergence) and husks of plants with new *B′* alleles. The DNA was digested with *Eco*RI plus *Sau*3AI or *Mbo*I, and DNA blot analyses were performed using all four probes shown in Figure 4. In the regions flanking the repeats, the new *B′* samples (9 samples from 7 individuals) showed the same methylation patterns as those observed with *B′* and *B-I* samples (0/9 were methylated at probe A, 3/9 were methylated at probe B, 1/9 had additional methylation at probe D). All nine new *B′* samples showed methylation of the third *Sau*3AI site in the repeats as seen with both *B′* and *B-I*. Interestingly, seven out of the nine new *B′* DNA samples showed methylation of the second *Sau*3AI site in the repeat, characteristic of the *B-I* pattern (Fig. 4, 376- and 853-bp fragments). There was variation in how many of the repeats were methylated at the second *Sau*3AI site in both new *B′* and *B-I*, but the variation was similar. We calculated the intensity of the 853-bp fragment and divided it by the total intensity (853 + 779 bp) in the 27 *B-I* and 7 new *B′* samples with substantial methylation at the second *Sau*3AI site. The ratios were 0.29 ± 0.08 and 0.3 ± 0.19 for *B-I* and new *B′* alleles, respectively. The remaining two new *B′* samples were much less methylated, but a slight hint of the 853-bp fragment characteristic of *B-I* was seen (the ratio of 853/(853 + 779) was 0.09 for both, whereas the ratios of the three *B-I* samples with low methylation were 0.04, 0.04, and 0.06). All plants had *B′* pigment levels throughout, suggesting that both alleles in most cells were *B′*. Thus, the *B′* epigenetic state can be established before the distinct methylation pattern, which indicates that the methylation reflects the epigenetic state rather than causes it.

Repeat methylation pattern in recombinants

To determine if the number of repeats or the nature of the flanking DNA affects the methylation pattern within the repeats, the same DNA methylation analyses were performed on DNA samples from tissues of 44 plants homozygous for eight different recombinants with recombination breakpoints within the repeats, upstream or downstream of the repeats. We tested the paramutable and paramutagenic alleles of *BI‖BP-D1* and *BI‖BP-D2* and four recombinants from previous experiments with *B-Peru* and *B′* (Stam et al. 2002). These were paramutagenic alleles with seven repeats, *B′‖BP-pg1*, *B′‖BP-pg2*, *BP‖B′-pg1*, and *BP‖B′-pg2*. With the probes flanking the repeats (Fig. 4, probes A, B, and D), all alleles showed the same methylation pattern previously observed. With the exception of *BI‖BP-D1*, the correlation between the repeat methylation pattern and the epigenetic state was maintained in the recombinants: if the allele was paramutable it had the *B-I* repeat methylation pattern, if it was paramutagenic it had the *B′* repeat methylation pattern.

The exceptional *BI‖BP-D1* allele has three tandem repeats and becomes paramutagenic (*BI‖BP-D1′*) when crossed to *B′*. However, the paramutagenic state is unstable as compared with alleles with five (*BI‖BP-D2′*) or seven (*B′*) repeats. We tested 14 samples from 11 *BI‖BP-D1* individuals in the *B-I* paramutable state: all showed the *B′* methylation pattern. We tested 11 samples from 8 *BI‖BP-D1′* paramutagenic plants, which had been carried with *B′* for two generations: 9 samples showed the *B′* methylation pattern; however, 2 samples showed the *B-I* methylation pattern. These individuals were also an exception to the rule that there were no methylation differences between leaves and husks: all 8 young leaf samples had the *B′* pattern, whereas 2 of the 3 husk samples showed the *B-I* pattern. Thus, in two out of three plants examined, the methylation pattern changed during development. One possibility is that fewer repeats reduces the stability of the methylation state. Alternatively, this allele may be in an epigenetic state distinct from *B′* or *B-I*.

B-I *has a more open chromatin structure than* B′

Altered chromatin structure frequently correlates with different epigenetic expression states. Previously we used a DNase I hypersensitivity assay on *B′* and *B-I* nuclei to compare the chromatin structure around the transcription start site (Chandler et al. 2000). In that experiment, nuclei were isolated from mature sheath tissue of *B′* and *B-I* plants, which show clear pigment differences. We observed increased hypersensitivity at a site near the transcription start in *B-I* nuclei relative to *B′* nuclei.

To determine if there were chromatin differences within the region required for paramutation, the same DNA blot was hybridized with several probes within and flanking the repeated region (Fig. 5). The 8.3-kb fragment spanning the repeat (Fig. 5, probe C) was more sensitive to DNase I in *B-I* nuclei than in *B′* nuclei, suggesting that *B-I* has a more open chromatin structure in this region compared with *B′* (Fig. 5). To determine if this difference was present outside the repeat region, the blot was stripped and rehybridized with probes B and E,

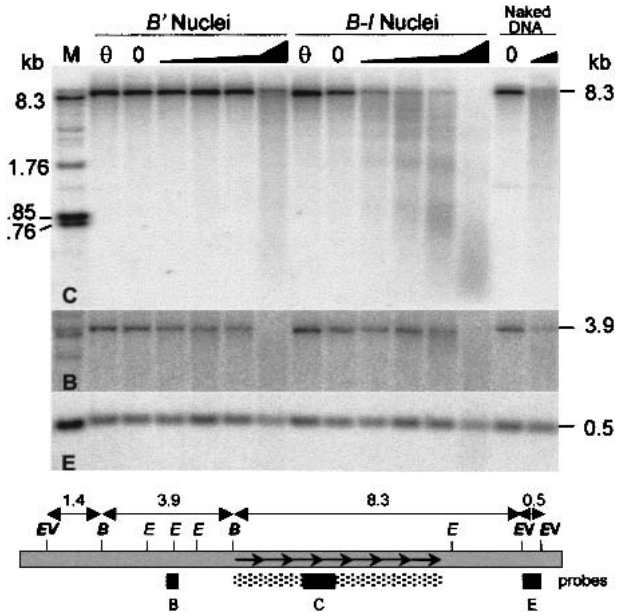

Figure 5. The tandem repeat region shows DNase I hypersensitivity in *B-I* chromatin. Nuclei derived from *B′* and *B-I* sheaths were treated with increasing amounts of DNase I (0.05, 0.2, 0.5, and 8 units of DNase I/mL; indicated by wedge); naked DNA was treated with no DNase I or 0.003 units of DNase I/mL. No DNase I was added to the nuclei followed by no incubation (lane θ) or incubation (lane *0*). All incubations were for 5 min. DNA was isolated, cut with *Eco*RV and *Bam*HI, and blotted. The restriction map shows the region analyzed and the probes used (B, C, and E); the stippled bar shows the region recognized by probe C. Arrows indicate the *Eco*RV–*Bam*HI fragments expected if the fragment is not cut by DNase I. The marker lane (M) contains a pool of *B-I* genomic DNA cut with *Eco*RV and one of the following enzymes: *Bam*HI, *Pst*I, *Xba*I, and *Bst*EII. The restriction sites are (B) *Bam*HI; (E) *Eco*RI; and (EV) *Eco*RV.

which recognize regions upstream and downstream of the repeated sequences (Fig. 5). Both regions were equivalently resistant to DNase I digestion in *B′* and *B-I* nuclei. Thus, differential sensitivity between *B′* and *B-I* was only detected in the region containing the repeats.

Newly formed B′ *alleles have a chromatin structure intermediate between* B′ *and* B-I

New *B′* alleles often retain the *B-I* methylation pattern, suggesting that the *B′* epigenetic state is established earlier than the methylation changes. To address whether the chromatin structure of new *B′* alleles reflected the parental *B-I* or the *B′* pattern, another DNase I assay was performed. This experiment used nuclei isolated from young sheaths and leaves surrounding the shoot meristem of *B′*, *B-I*, and new *B′* plants. These young tissues were green because *B′* and *B-I* were not yet expressed, allowing us to determine the chromatin state prior to transcription of *B′* and *B-I*.

To map hypersensitive sites within the repeats, we hybridized with probe D1, a single-copy probe located just downstream of the repeats and just upstream of the *Eco*RI site (Fig. 6). The DNase I sensitivity of the chromatin in the new *B′* repeats was intermediate between that of *B′* and *B-I* (Fig. 6). The non-DNase I controls

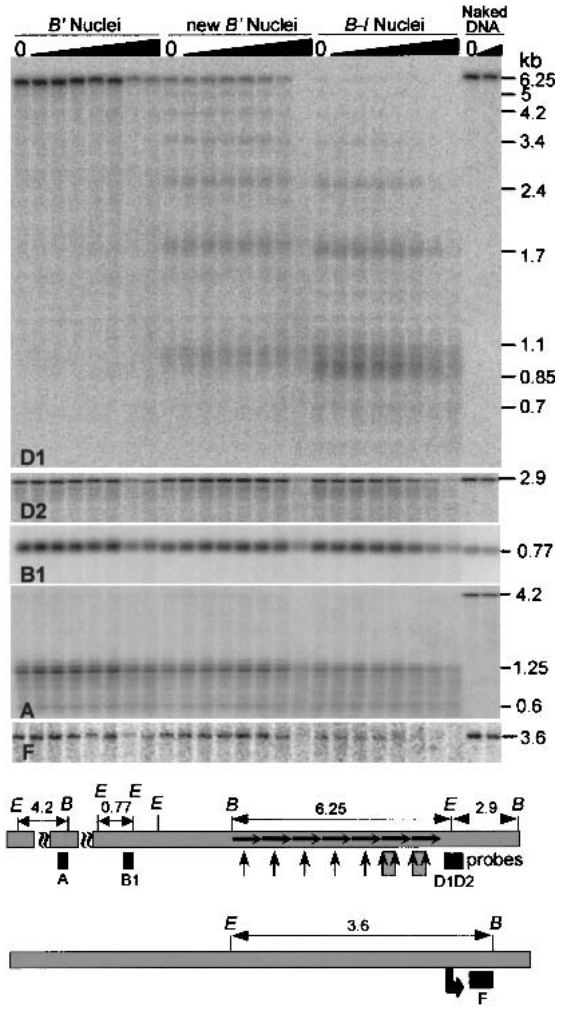

Figure 6. The tandem repeats in new *B′* chromatin show an intermediate chromatin structure relative to *B-I* and *B′*. Nuclei from *B′*, new *B′*, and *B-I* tissue were treated with increasing amounts of DNase I (0, 0.05, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 units of DNase I/mL; indicated by wedge). Naked DNA was treated with 0 or 0.005 units of DNase I/mL. All incubations were for 6 min. The lack of digestion of naked DNA and cutting of DNA in nuclei only at the highest concentrations used indicated that the DNase I was not very active. The 0 DNase I control shows that most digestion was by endogenous nucleases. DNA was isolated, cut with *Eco*RI and *Bam*HI, and blotted. The maps show the regions analyzed and the probes used (A, B1, D1, D2, and F). Arrows indicate the *Eco*RI–*Bam*HI fragments expected if the fragments are uncut by nucleases. The probes monitor the same general regions as in Fig. 5, but are at the end of each restriction fragment to map hypersensitive sites. Endogenous nuclease-hypersensitive sites are indicated with vertical arrows beneath the restriction map. The restriction sites are (B) *Bam*HI and (E) *Eco*RI.

indicated that primarily endogenous nucleases rather than the exogenously added DNase I are responsible for the digestions. Nonetheless, comparison of the hybridization pattern of probe B1 hybridizing upstream of the repeats with that of the probe monitoring the repeats makes clear that the endogenous nucleases preferentially act on the repeated region in both *B-I* and new *B′* nuclei. *B′* nuclei also contained endogenous nucleases because probe A (Fig. 4, unmethylated, gene-like sequence upstream of the repeats) showed similar digestion in both *B′* and new *B′* samples (Fig. 6), yet the repeats in *B′* are not cut by the endogenous nucleases. In *B-I* nuclei the gene-like region monitored with probe A is slightly more digested with endogenous nucleases. This could mean that in *B-I* nuclei this region has a more sensitive chromatin structure, or that there were slightly more endogenous nucleases.

The digestion pattern reflecting multiples of ~850 bp seen in *B-I* and new *B′* chromatin is consistent with nucleases cutting in each repeat (indicated with arrows in Fig. 6). In the naked DNA control, the repeats were not cut, but at the concentrations used the DNase I had little activity. If the pattern of cutting within each repeat is caused by the repeat DNA structure, we should see the pattern in naked DNA. We did extensive DNase I titrations on naked genomic and plasmid DNA containing the repeats. We only observed general degradation in naked DNA; no repeat ladder was observed (data not shown), indicating that the pattern of hypersensitive sites in the repeated region is mediated through chromatin structure.

We next tested for hypersensitive sites around the transcription start site by hybridizing with the F probe (Fig. 6); this region was sensitive in *B-I* and equivalently resistant in *B′* and new *B′* nuclei. Thus, in young, unpigmented tissue, the chromatin structure spanning the transcription start site predicts the transcription state that will occur: *B-I* will be more transcribed and is more sensitive than *B′* and new *B′*.

Discussion

Using fine-structure recombination mapping, we localized the region within *B-I* required for enhancer activity, paramutability, and paramutagenicity to a 6-kb region, located ~100 kb upstream of the transcription start site. This region has an identical DNA sequence in *B-I* and *B′*, and consists mostly of seven tandem repeats of a 853-bp sequence unique to this region of the genome. The fact that *B-I* and *B′* have identical sequences within the region required for paramutation establishes that *B′* paramutation is an epigenetic phenomenon, in spite of the fact that *B′* is extremely stable. Comparison of recombinant alleles with *B-I* sequences upstream, *B-Peru* sequences downstream, and one, three, or five repeats, each allele possessing all or most of the *B-Peru* sequence polymorphisms in the repeat, shows that multiple repeats are required for paramutation and enhancer activity. The recombinant with three repeats showed a reduced stability of the paramutagenic state, whereas the recombinant with five repeats was fully paramutagenic. In contrast, the recombinant with one repeat could not participate in paramutation and had no enhancer activity. Further experiments will be required to determine if multiple repeats are sufficient for both activities, or if the repeats are interacting with other sequences shared between *B-I* and *B-Peru*.

The region required for *b1* paramutation is unique in the maize genome, which suggests that some structural feature may be shared with other maize loci that undergo paramutation. Inverted and direct repeats of transgenes are often, but not always, correlated with silencing (for reviews, see Henikoff 1998; Wolffe and Matzke 1999; Muskens et al. 2000). In many cases the silencing is post-transcriptional and associated with inverted repeats that produce double-stranded RNA homologous to the transcribed region. Transcriptional gene silencing is usually associated with repeated promoter sequences, but the repeats often span the transcribed region as well. Simply the presence of repeats is not necessary for paramutation, because a single-copy transgene insert can efficiently mediate paramutation in Petunia (Meyer et al. 1993). Although the strength of paramutation at *r1* correlates with the number of repeats (Kermicle et al. 1995), the *r1* repeats are very different from what we observe at *b1*. In two paramutagenic *r1* alleles, each repeat is at least 13 kb (Eggleston et al. 1995) or 26 kb (Panavas et al. 1999) long, spanning the transcribed region and thousands of base pairs of flanking DNA. In contrast to the repeats correlated with transgene silencing and *r1* paramutation, the *b1* tandem repeats are 100 kb upstream and share no homology with the promoter-proximal or transcribed regions.

Specific sequences required for paramutation have been identified for paramutation mediated by transgenes containing *p1* regulatory sequences. A 1.2-kb *p1* fragment, containing an enhancer, with ~78 bp 100% identical to the *p1* transcript, and present in a multicopy transgene array, paramutates the endogenous allele (Sidorenko and Peterson 2001). Another enhancer from the *p1* promoter in a multicopy transgene array has no paramutagenic activity (Sidorenko and Peterson 2001). It is not known whether the enhancer or transcript homology regions are important contributors nor whether repeats are required. Also, the sequences required for paramutation within the endogenous allele are not known. Paramutation mechanisms are shared at several maize genes because a mutation that prevents paramutation at *b1* also prevents paramutation at two other loci (Dorweiler et al. 2000). We suggest that the common denominator is heritable alterations in chromatin structure; there are no common sequences and no clear correlation with the types of repeats among these maize genes.

Comparison of the DNA methylation pattern and chromatin structure of the *B-I* and *B′* repeat region indicates that in the high-expressing *B-I* allele the repeats are more hypersensitive to nucleases, but they are more methylated relative to the repeats in the low-expressing *B′* allele. Correlations between *r1* paramutation and

methylation have been seen, but the correlation is in the opposite direction (the more silenced state is more methylated), and the sequences being monitored have not been shown to be required for paramutagenicity (for review, see Chandler et al. 2000). Possibly at *b1*, a repressor protein binds unmethylated sites in *B′* repeats. Alternatively, the binding of an activator could cause the strong hypersensitivity of the repeats in *B-I*. Potentially, the more open chromatin structure of *B-I* enables the methylation machinery better access, resulting in the methylation increases relative to *B′*. Intriguingly, in the three-repeat allele the repeat methylation pattern is unstable, as is the paramutagenic ability of this allele.

Our studies with new *B′* alleles deriving spontaneously from *B-I* parents show it often takes more than one generation to go from the *B-I* to the *B′* methylation pattern. This indicates that the repeat methylation pattern at the second *Sau*3AI site follows the establishment of the epigenetic state rather than causes it. This finding also suggests the increased methylation in *B-I* does not directly affect the binding of regulatory proteins required for establishment and maintenance of the paramutagenic state. The repeat chromatin structure of new *B′* alleles, assayed with endogenous nucleases, had a sensitivity intermediate to that of *B-I* and *B′*, consistent with the observation that, on average, new *B′* alleles are slightly more pigmented relative to alleles that have been *B′* for multiple generations (Patterson and Chandler 1995). The differing sensitivity between *B′* and *B-I* is observed in tissues developmentally earlier than the onset of transcription, suggesting that chromatin structure could be the heritable imprint that distinguishes the distinct transcription states. Further studies will be required to determine whether the imprint is mediated by DNA methylation, histone modification, or other chromatin modifications.

This is the first example in plants of regulatory sequences located far upstream of the coding region of a gene. Based on paradigms that derive from studies in mammals, we hypothesize that the enhancer exists in two states, functional (*B-I*) and nonfunctional (*B′*), possibly because it is part of or adjacent to boundary/insulator sequences that exist in distinct states. The LCR (locus control region) of the β-*globin* loci in mammals contains hypersensitive sites far upstream from the globin genes, some of which function as insulator/boundary elements, others as enhancers (Bell et al. 2001). In mice, an enhancer ~10 kb from *H19* and ~100 kb from *Igf2* functions on one of the two genes depending on the chromatin structure and methylation status of the ICR region between the two genes (Bell and Felsenfeld 2000; Hark et al. 2000). The CTCF protein binds to the unmethylated and nuclease-hypersensitive ICR, which then functions as a boundary, preventing the enhancer from acting on *Igf2*. When the ICR is methylated and nuclease-insensitive, the enhancer acts on *Igf2*. Decreased DNA methylation in the ICR correlates with a more open chromatin structure, opposite of what is observed at *B′* and *B-I*. A striking similarity between our results and imprinted genes is that imprinted genes are associated with tandem repeat sequences in the differentially methylated ICR. These are generally short (25–120 bp; Barlow 1995), but at the human *H19* locus there are several copies of two different ~400-bp repeats (Jinno et al. 1996). The repeat sequences associated with imprinted genes are also unique to one region in the maize genome. Further dissection of the 6-kb *B-I* fragment using transgenic approaches will address if the enhancer activity and postulated boundary or insulator elements are distinct or overlap. Identification of the proteins encoded by genes required for paramutation (Dorweiler et al. 2000; Hollick and Chandler 2001) may also help to support or refute this model.

The most intriguing aspect of paramutation is that *trans* interactions alter the transcription state of one allele. Mitotically heritable *trans*-sensing phenomena in *Drosophila* provide paradigms (Henikoff and Comai 1998; Wu and Morris 1999), because in plants, mitotically heritable changes in expression are often transmitted to progeny as germ cells differentiate from somatic cells late in development. Many animals and insects set germ cells aside early in development. Pairing may mediate allele interactions, as observed with many *trans*-sensing phenomena, in which translocations that disrupt somatic pairing disrupt these phenomena. Translocation of *B-I* or *B′* to other chromosome arms does not disrupt paramutation (Coe 1966), but the translocated regions are large such that meiotic pairing (and potentially somatic pairing) is not disrupted. Although there is no evidence for homolog synapsis in somatic plant cells, transient interactions are all that are needed, because once the *B′* state is established, it is maintained independent of what other allele is on the homologous chromosome. There are also examples of transvection in *Drosophila* that do not require stable synapsis in somatic tissues (Hopmann et al. 1995). Subnuclear localization could also be involved in paramutation. The heterochromatic insertion at $brown^D$ interacts with centromeric heterochromatin, sequestering the wild-type gene into a specific heterochromatic compartment and thus affecting its expression (Csink and Henikoff 1996). Possibly *B′* and *B-I* are usually in distinct subnuclear domains and allele interactions result in similar compartmentalization.

An alternative model to DNA interactions is DNA/RNA interactions. X-Chromosome inactivation involves the stabilization of an untranslated RNA, which appears to be both necessary and sufficient to confer a chromatin-based mechanism of inactivation on adjacent sequences (Boumil and Lee 2001). Although usually not meiotically heritable, RNA silencing provides another paradigm because it occurs in *trans*, is mediated by small RNAs, and is often associated with methylation of homologous DNA (for review, see Wolffe and Matzke 1999; Jones et al. 2001). Possibly RNA molecules mediate *trans* interactions, which establish a chromatin state that is epigenetically maintained.

Paramutation and other examples of homology-dependent gene silencing have been postulated to represent cellular mechanisms for protection against invasive DNA (Matzke et al. 1996; Yoder et al. 1997). The ma-

chinery that mediates paramutation may function to identify and maintain chromatin boundaries between genes and nearby repetitive sequences, or to provide an adaptive mechanism for transmitting favorable expression states to progeny (Chandler et al. 2000). Our identification of the sequences required for paramutation, coupled with *trans*-acting mutants altered in paramutation, provide the tools to fully understand the underlying mechanisms and cellular function in the near future.

Materials and methods

Maize nomenclature

In maize nomenclature, a gene is designated with lowercase italics, and specific alleles are indicated by an allele designation separated from the gene designation with a hyphen. Dominant alleles are indicated by an uppercase gene designation (*B-I*) and recessive alleles by a lowercase gene designation (*b-K55*; http://www.agron.missouri.edu/maize_nomenclature.html).

Plant stocks

All plant stocks had dominant functional alleles for all genes encoding anthocyanin biosynthetic enzymes. Stocks containing various *b1* alleles maintained in the Chandler laboratory were originally obtained from various sources: *B-I* (W23 background), *B′*, and *b-K55* (K55 background) were from E.H. Coe, Jr. (University of Missouri, Columbia); *B-Peru* (W22 background) was from M.G. Neuffer (University of Missouri); and *bm-1* contains a *Ds* transposon in the *B-Peru* coding region (Clark et al. 1990).

Recombinant screen

When indicating genotypes, a single allele indicates a homozygote, and in heterozygotes the two alleles are separated by a slash (/). To identify Interval I and II recombinant alleles between *B-I* and *B-Peru*, we made use of the phenotypic marker *gl2* (*glossy2*), 19 cM upstream of the *b1* transcription start site (http://www.agron.missouri.edu/maps.html), and the tightly linked gene *tmp*, 0.18 cM upstream of the *b1* transcription start site (Stam et al. 2000). Wild-type and mutant *glossy2* phenotypes result in different juvenile waxes, visually scored in young seedlings. The *gl2 B-I* and *Gl2 B-Peru* stocks were polymorphic within the third intron of the *tmp* gene, *tmp-I* and *tmp-P*, respectively.

To isolate recombinants between *B-I* and *B-Peru*, *gl2 B-I*/*Gl2 B-Peru* plants were crossed with *gl2 b-K55* (colorless seeds, *tmp-I*) or *bm-1* (colorless seeds, *tmp-P*). To isolate *B-I* recombinants, colorless seeds from the *gl2 b-K55* cross were planted in trays, *Gl2* seedlings were transplanted, and the resulting plants were pooled and screened for *tmp* markers as described (Stam et al. 2002). To isolate *B-Peru* recombinants that gained enhancer sequences, dark seeds from the *gl2 b-K55* cross were planted either directly in the field or in trays, and resulting *gl2* seedlings were then transplanted. When mature, plants were scored for their pigment phenotype, and plants with vegetative pigment darker than *B-Peru* were individually tested for loss of *tmp-P*. To isolate *B-Peru* recombinants with breakpoints upstream of the enhancer sequences, dark seeds from the cross with *bm-1* were planted in trays. Then *gl2* seedlings were transplanted and screened in pools using PCR to specifically amplify a 543-bp *B-I* fragment between 108.5 and 109 kb upstream of the *B-I* transcription start site (VC90–VC57). As an internal control, a 963-bp upstream *B-Peru* fragment (VC81–VC80) was amplified; VC90, 5′-GGATATTCATGCATAACCATTGCTTC-3′; VC57, 5′-GTTGCTACTGTTACACCAGTTTGC-3′; VC81, 5′-CGGCTCCCCTGCGGATCCTC-3′; VC80, 5′-CCATATTCA CGCTAGTCCAAATCG-3′. The PCR conditions were 1× PCR and 1× Q buffer (both QIAGEN), 0.1 mM dNTPs, 0.8 mM of each primer, 1 unit of Taq DNA polymerase, reaction volume of 25 µL; 2 min at 94°C, 35 times (1 min at 94°C, 1 min at 61°C, 1 min 20 sec at 72°C), and then 10 min at 72°C, up to 1 h at 4°C.

Cloning and sequence analyses of b *alleles*

The comparable region of the *B′* sequence 94–110 kb upstream of the transcription start was PCR-amplified from *B-I*, *B-P*, and *b-K55* DNA using primers designed from the previously determined *B′* sequence (Stam et al. 2002). Resulting fragments were cloned into pGEM-T Easy (Promega) using standard molecular techniques. For each fragment, plasmid DNA from five to eight clones was pooled and sequenced. To verify that the amplified fragments were physically linked in the genome, fragments overlapping two contiguous fragments were amplified, cloned, and sequenced. All sequences of a specific allele were assembled with FAKtory, a sequence assembly program (http://bcf.arl.arizona.edu/faktory). Accession numbers are AY078064 (*b-K55*), AF483658 and AF484216 (*B-Peru*), and AF483657 (*B-I*).

The repeat sequence was analyzed with a variety of computer programs. No significant similarity to the nr database using TBLASTX (Altschul et al. 1997) was found. For gene annotation we used: GENESCAN using Maize matrix at http://genes.mit.edu/GENSCAN.html; FGENESH using plant model at http://genomic.sanger.ac.uk/gf/gf.shtml; and GeneMark Eukaryotic model at http://opal.biology.gatech.edu/GeneMark/eukhmm.cgi. Two programs, REPuter (http://bibiserv.techfak.uni-bielefeld.de/reputer) and Tandem Repeats Finder (http://c3.biomath.mssm.edu), were used to search for small inverted and direct repeats. To search for ARS we used the FindPatterns program from GCG (University of Wisconsin). To search for MARs we used SMARTest (http://www.genomatix.de/cgi-bin/smartest_pd/smartest.pl).

Genomic DNA analyses

To confirm sequence contigs and examine DNA methylation patterns, ~4 µg of genomic DNA from *B′*, *B-I*, *B-Peru*, and *b-K55* leaves (Dellaporta et al. 1983) was digested with several enzymes and enzyme combinations according to manufacturers' specifications, size-fractionated by electrophoresis in 0.5× TBE agarose gels, followed by DNA blot analyses (Stam et al. 2002).

DNase I hypersensitivity assay

Nuclei were isolated from ~7-week-old plants, from the most mature, pigmented, outer sheaths (Fig. 5); and from green, inner sheaths and associated leaves surrounding the shoot meristem (Fig. 6). Nuclei were prepared (Steinmuller and Apel 1986) with the buffer modified to contain 5 mM EDTA, 0.5 mM EGTA, and 0.1 mM PMSF. Aliquots of 1 million nuclei from each genotype were incubated with increasing concentrations of DNase I from Roche (Chandler et al. 2000).

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